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Secondary Structures of Chloroplast *trn*L Intron in Dipterocarpaceae and its Implication for the Phylogenetic Reconstruction

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Unambiguous insertion-deletion events were previously identified in *trn*L intron of 110 species of subfamily Dipterocarpoideae (Dipterocarpaceae). These indels are associated with the formation of four stem loop structures and featuring characteristic for generic/infra-generic level depended upon which taxonomic classifications are followed. Phylogenetic analyses were performed by including and excluding these structures to examine the robustness of resulted topologies. Results indicated that inclusion of such structures yielded more resoved topologies, and that none of the stemloop structures were homoplasious. Results of this present study was also in agreement with the previous molecular phylogenetic studies that using several genes of cp genomes in that tribe Dipterocarpae was polyphyletic by the placement of all members of the genus *Dipterocarpus* within tribe Shoreae, and that tribe Shoreae was a potential monophyletic group. The phylogenetic relationships between variable genera of *Hopea* and *Shorea* was also in accordance to earlier studies that suggested a potential monophyly of the two with inclusion of *Parashorea* and *Neobalanocapus heimii*. Genera that were recived strong branch support (*Dipterocarpus, Dryobalanops, Vatica,* and *Stemonoporus*) possessed certains indels exclusive to each and this may contributed to the monophyletic nature of these genera.

Key words: secondary structures, dipterocarpaceae, *trn*L, intron, phylogeny

INTRODUCTION

The *trn*L-F of chloroplast genome of land plants consists of the transfer RNA genes $trnL_{uaa}$ and $trnF_{gaa}$ arranged in tandem and separated by noncoding spacer regions. The region is positioned in large single copy region, approximately 8 kb downstream of *rbc*L. The conserved nature of *trn*L-F region made the design of plant universal primers possible (Tarbelet *et al.* 1991), thus this region has become one of the most widely used chloroplast markers for phylogenetic analyses in plants (Borsch *et al*. 2003; Hamilton *et al.* 2003; Pirie *et al.* 2007; Shaw *et al.* 2007; Koch *et al.* 2007). The *trn*L gene is part of *trn*L-F region of chloroplast genome that split by group I intron, the intergenic spacer and *trn*F exons (Figure 1) and is co-transcribed (Bakker *et al*. 2000). The intron is positioned between the U and the A of the UAA anticodon loop. Secondary structures within the *trn*L intron is important because the function of the transfer RNA for which the *trn*L gene codes is related to it and that of the intron within it (Pirie *et al.* 2007). Hence, deduction of positional homology -which is the most important part for the phylogenetic reconstruction- of the structure is important during the process of DNA alignment.

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Sequences from *trn*L-F regions in combination with other cp and nuclear genomes have been used in phylogenetic reconstruction of Dipterocarpaceae (Tsumura *et al.* 1996; Kajita *et al.* 1998; Dayanandan *et al.* 1999; Kamiya *et al.* 2005; Yulita *et al.* 2005; Gamage *et al*. 2006), population genetic study (Aoki *et al.* 2003) and even DNA *barcoding* (Tarbelet *et al*. 2007). However, none of the studies have examined the evidence of secondary structure of *trn*L intron into detail. Four unambiguous indels were previously described in Dipterocarpaceae (Yulita 2007). These indels made stem loop structures located at position 70-105 bp (Stem Loop/SL 1), 153-171 (SL 2), 257-328 (SL 3), and 360-386 (SL 4) (Figure 2). Large indels have mostly been excluded from the data set (Koch *et al.* 2007) since it may provide 'noise' within the phylogenetic analysis, although structural mutation built from indels can be reliable markers for phylogenetic reconstruction in

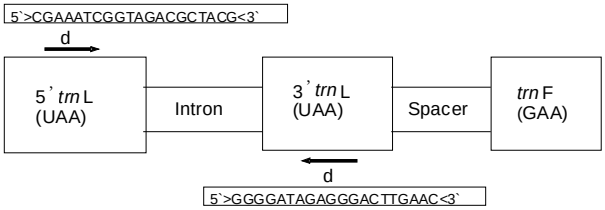

Figure 1. Diagram of *trn*L-F gene with primer sequences of intron *trn*L (c and d) (after Yulita 2007).

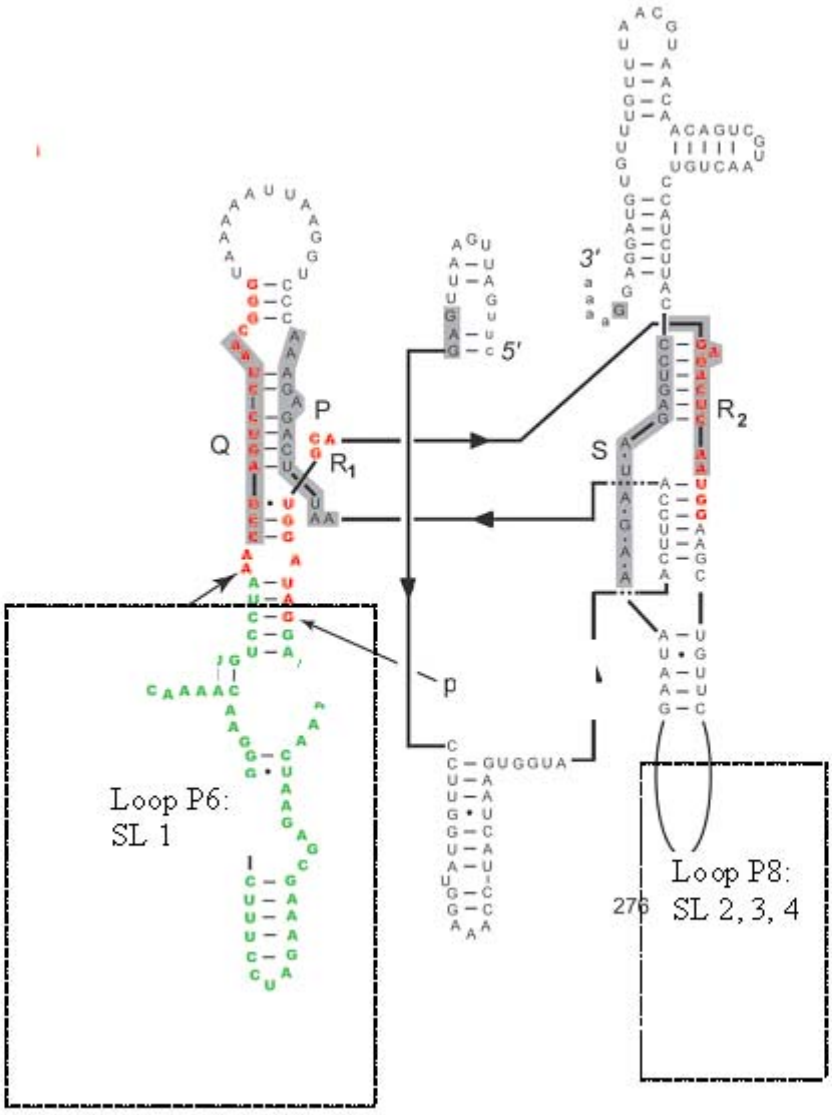

Figure 2. Secondary structure of *trn*L intron of Dipterocarpaceae that was modified from *Nymphaea odorata* (Tarbelet *et al*. 2007). Location of *stem loop* 1 (SL1) was in loop P6, locations of stem loop 2, 3, dan 4 (SL 2, 3, 4) were in loop P8 (after Yulita 2007).

some plant groups (Soltis *et al.* 1992). Examination for these structures, however, suggested that these have implications on taxonomic diagnostic characters as certain indels were possessed by certain taxa in Dipterocarpaceae. This present study was aimed to test the utility of the indels in assessing phylogenetic relationships among species of Dipterocarpaceae.

MATERIALS AND METHODS

The *trn*L intron sequences of 110 species of 14 genera of Dipterocarpaceae were obtained from the genbank database (http://www.ncbi.nlm.nih.gov/). The list of genebank accession number in those samples is detailed in Table 1. The raw sequences were aligned using Clustal X (Thompson *et al.* 1997) and eyed refined to determine the positional homology. The existence of inverted repeat was examined by GENETYX and eyed refined. These structures were particularly built in regions that have long repeat, insertions and deletions, and hotspot for base substitution.

Two cladistic analyses were performed using PAUP (Swofford 1998) by including and excluding secondary structures. The optimal tree was estimated using a heuristic search strategy with maximum parsimony criterion. A hundred replicate searches were conducted using random addition to search across multiple islands of trees. This strategy was used for all final tree searches. Initial MAXTREES was set to 230,000 (auto-increased by 100). Tree Bisection Reconnection (TBR) branch-swapping was used, with the steepest descent option off and using ACCTRAN (Accelerated Transformation) optimisation. The MULPARS (multiple parsimonious trees) option was on and minimum branches of zero were collapsed. Ten equally parsimonious trees were held following each replicate.

Table 1. Spesies samples and Genbank accession numbers

Spesies	Abreviation	Genbank accession number
Anisoptera laevis	ALAEV	AB006387
Anisoptera oblonga	AOBLO	AB006388
Cotylelobium malayanum	CMALA	AB006389
Cotylelobium scabriusculum	CSCRO	AB246545
Dipterocarpus alatus	DALAT	AB246603
Dipterocarpus confertus	DCONF	AY026528
Dipterocarpus cornutus	DCORN	AB246602
Dipterocarpus glandulosus	DGLAN	AB246607
Dipterocarpus hispidus	DHISP	AB246606
Dipterocarpus insignis	DINSI	AB246605
Dipterocarpus kerrii	DKERI	AB006392
Dipterocarpus retusus	DRETU	AY026529
Dipterocarpus zeylanicus	DZEYL	AB246604
Dryobalanops aromatica	DRARO	AY026530
Dryobalanops lanceolata	DRLAN	AY026531
Dryobalanops oblongifolia	DOBLO	AB006395
Hopea apiculata	HAPIC	AY026532
Hopea brevipetiolaris	HBREV	AY026533
Hopea celebica	HCELE	AY026534
Hopea celtidifolia	HCELT	AY026535
Hopea cernua	HCERN	AY026536
Hopea cordifolia	HCORD	AY026537
Hopea discolor	HDISC	AB246588
Hopea dryobalanoides	HDRYO	AY026538
Hopea ferruginea	HFERR	AY026594
Hopea helferi	HHELF	AB246587
Hopea jucunda	HJUCU	AY026540
Hopea latifolia	HLATI	AB246586
Hopea mengerawan	HMENG	AY026541
Hopea nervosa	HNERV	AB006401
Hopea nigra	HNIGR	AY026542
Hopea pierrei	HPIER	AY026543
Hopea pubescens	HPUBE	AY026544
Hopea subalata	HSUBA	AB246585
Hopea wightiana	HWIGH	AY026545
Monotes madagascariensis	MMADA	AB246608
Neobalanocarpus heimii	NHEMI	AB006400
Parashorea lucida	PLUCI	AB006399
Shorea acuminata	SACUM	AB006399
Shorea affinis	SAFFI	AB246601
Shorea assamica	SASSA	AB246583
Shorea balangeran	SBALA	AY026546
Shorea beccariana	SBECC	AY026547
Shorea bracteolata	SBRAC	AB006398
Shorea bullata	SBULLA	AB246565
Shorea congestiflora	SCONG	AB246593
Shorea cordifolia	SCORD	AB246592
Shorea curtisii	SCURT	AB246563
Shorea disticha	SDIST	AB246595
Shorea dyeri	SDYER	AB246576
Shorea elliptica	SELLI	AB246574
Shorea exelliptica	SEXEL	AY026548
Shorea faguetiana	SFAGU	AY026549
Shorea fallax	SFALL	AB246564
Shorea foxworthyi	SFOXW	AY026550

Table 1. Continue

Spesies	Abreviation	Genbank accession number
Shorea gardneri	SGARD	AB246598
Shorea guiso	SGUIS	AY026551
Shorea hopeifolia	SHOPE	AY026552
Shorea isoptera	SISOP	AY026553
Shorea johorensis	SJOHO	AY026555
Shorea kunstleri	SKUNS	AY026556
Shorea laevis	SLAEV	AY026557
Shorea leprosula	SLEPR	AY026558
Shorea lissophylla	SLYSS	AB246577
Shorea longisperma	SLONG	AY026559
Shorea macrophylla	SMACR	AY026560
Shorea macroptera	SMACT	AB006396
Shorea materialis	SMATE	AY026561
Shorea maxima	SMAXI	AY026562
Shorea maxwelliana	SMAXW	AY026563
Shorea megistophylla	SMEGI	AB246594
Shorea multiflora	SMULT	AY026565
Shorea ovalis	SOVAL	AY026566
Shorea palembanica	SPALE	AY026567
Shorea pallescens	SPALL	AB246578
Shorea parvifolia	SFOLI	AY026568
Shorea parvistipulata	SPARV	AY026569
Shorea pilosa	SPILO	AY026570
Shorea pinanga	SPING	AY026571
Shorea quadrinervis	SQUAD	AB246566
Shorea richetia	SRICH	AY026572
Shorea roxburghii	SROXB	AY026573
Shorea scaberrima	SSCAB	AY026574
Shorea selanica	SSELA	AY026575
Shorea seminis	SSEMI	AY026576
Shorea singkawang	SSING	AY026577
Shorea smithiana	SSMIT	AY026578
Shorea splendens	SSPLN	AB246573
Shorea splendida	SSPLE	AY026579
Shorea stenoptera	SSTEN	AY026580
Shorea stipularis	SSTIP	AB246584
Shorea trapezifolia	STRAP	AB246596
Shorea virescens	SVIRE	AY026581
Shorea worthingtonii	SWORT	AB246599
Stemonoporus acuminatus	STACU	AB246552
Stemonoporus bullatus	STBUL	AB246556
Stemonoporus canaliculatus	STCAN	AB246555
Stemonoporus gilimalensis	STGIL	AB246553
Stemonoporus kanneliyensis	STKAN	AB246559
Stemonoporus lancifolius	STLAN	AB246560
Stemonoporus reticulatus	STRET	AB246557
Stemonoporus scalarinervis	STSCA	AB246554
Stemonoporus wightii	STWIG	AB246558
Upuna borneensis	UBORN	AB006391
Vateria copallifera	VCOPA	AB246561
Vateriopsis seychellarum	VSEYC	AB246562
Vatica affinis	VAFFI	AB246551
Vatica bella	VBELL	AB246546
Vatica chinensis	VCHIN	AB246550
Vatica coriacea	VCORI	AB246548

The character states were treated as unordered only (Fitch 1971). Statistical measures of the Consistency Index (CI), Homoplasy Index (HI) (Kluge & Farris 1994), Rescaled Consistency Index (RC), and Retention Index (RI) (Farris 1989) were also calculated. Clade support was estimated by performing 100 bootstrap replicates (Felsenstein 1985) by using 50% majority-rule of MPT input as trees but with MULPARS off. Definition of bootstrap supports were following Richardson *et al.* (2004):

50-74% represents weak support, 75-84% moderate support, 85-100% strong support.

RESULTS

Inclusion of Secondary Structures. The aligned sequences used for this study was 524 bp. The high content of adenine and thymine within *trn*L intron was therefore suggesting that this region was relatively A+T rich. The four stem loop structures present in intron *trn*L were consisted of seven indels: indel 1 was deletion of 5 bp within the loop of SL 1 (Figure 3), indel 2, 3, 4, and 5 were present in SL3 (Figure 4), and indels 6 and 7 were observed in SL 4 (Figure 5). SL 2, however, did not contain any indels. These seven indels were coded as additional characters, thus made up the total of 531 characters. Of these, only 59 were parsimony-informative characters.

A total of 107 of mostly parsimonius trees of 215 steps were obtained. The CI (0.83), RC (0.77), and RI (0.92) values suggest that the changes are mostly apomorphic, despite homoplasy occurring in 17% of the characters. Most of the clades were defined by apomorphic changes rather than synapomorphic changes. Apomorphic changes are mostly provided by base substitutions.

The cladogram (Figure 6) shows two paraphyletic groups with *Monotes madagascariensis* fall excluded from two groups. The first group is moderately supported (BSV of 81%) consisted of most member of tribe Dipterocarpeae except for *Dipterocarpus*. Of these members of tribe Dipterocarpae, only *Stemonoporus* and *Vatica* was supported 90 and 84% respectively.

The second main clade did not receive support from bootstrap. *Dipterocarpus* that was at the basal clade as the sister of Tribe Shoreae, containing *Dryobalanops*, *Parashorea*, *Neobalanocarpus heimii* and *Hopea-Shorea* clades. *Hopea* and *Neobalanocarpus heimii* formed a group probably monophyletic, while *Shorea* and *Parashorea* were scattered over the lineages. The only potential monophyletic group of *Shorea* was Section *Richetioides* (Yellow Meranti) and Section *Doona* (Sri-Lankan endemic).

Exclusion of Secondary Structures. Excluding the 4 SL characters resulted in 370 characters to which 265 characters are constant, 67 characters were parsimony-uninformative, and only 38 are parsimony informative characters. There were 1196 most parsimonius trees of 136 steps were obtained. The CI (0.6935), RC (0.7979), and RI (0.9275) values suggest that the changes are mostly apomorphic, despite homoplasy occurring in 14% of the characters. Most of the clades were defined by apomorphic changes rather than synapomorphic changes. Apomorphic changes were mostly provided by base substitutions.

The cladogram still showed similar grouping as of inclusion of indels. *Monotes madagascarensis* still form a single lineage. Two main paraphyletic groups were recognized whose divisions were almost in accordance to tribal divisions except for inclusion of *Dipterocarpus* spp. within Tribe Shoreae. Tribe Dipterocarpeae (B) was strongly supported (BSV

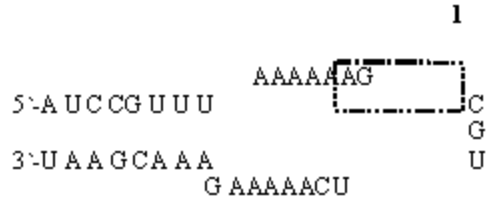

Figure 3. Structure of ste*m loop* 1 (70-105 bp). This model was derived from RNA sequence of *Neobalanocarpus heimii* (after Yulita 2007). Nucleotides in dotted box indicates location of indel 1.

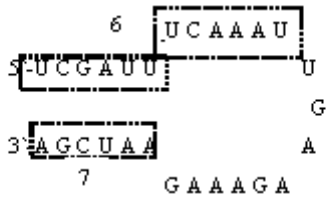

Figure 5. Structure of ste*m* loop 4 (360-386). This model was derived from RNA sequence of *Neobalanocarpus heimii* (after Yulita 2007). Nucleotides in dotted boxes indicate locations of indel 6 and 7.

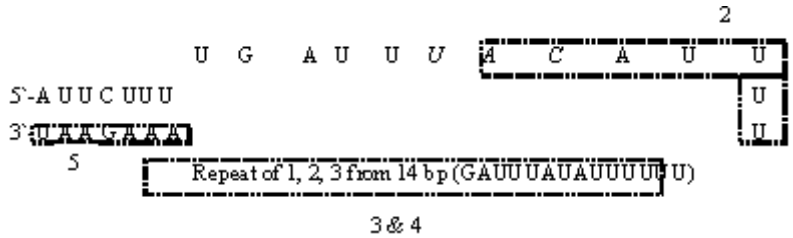

Figure 4. Structure of stem loop 3 (257-328). This model was derived from RNA sequence of *Dipterocarpus kerrii* (after Yulita 2007). Nucleotides in dotted boxes indicate locations of indel 2,3, and 4.

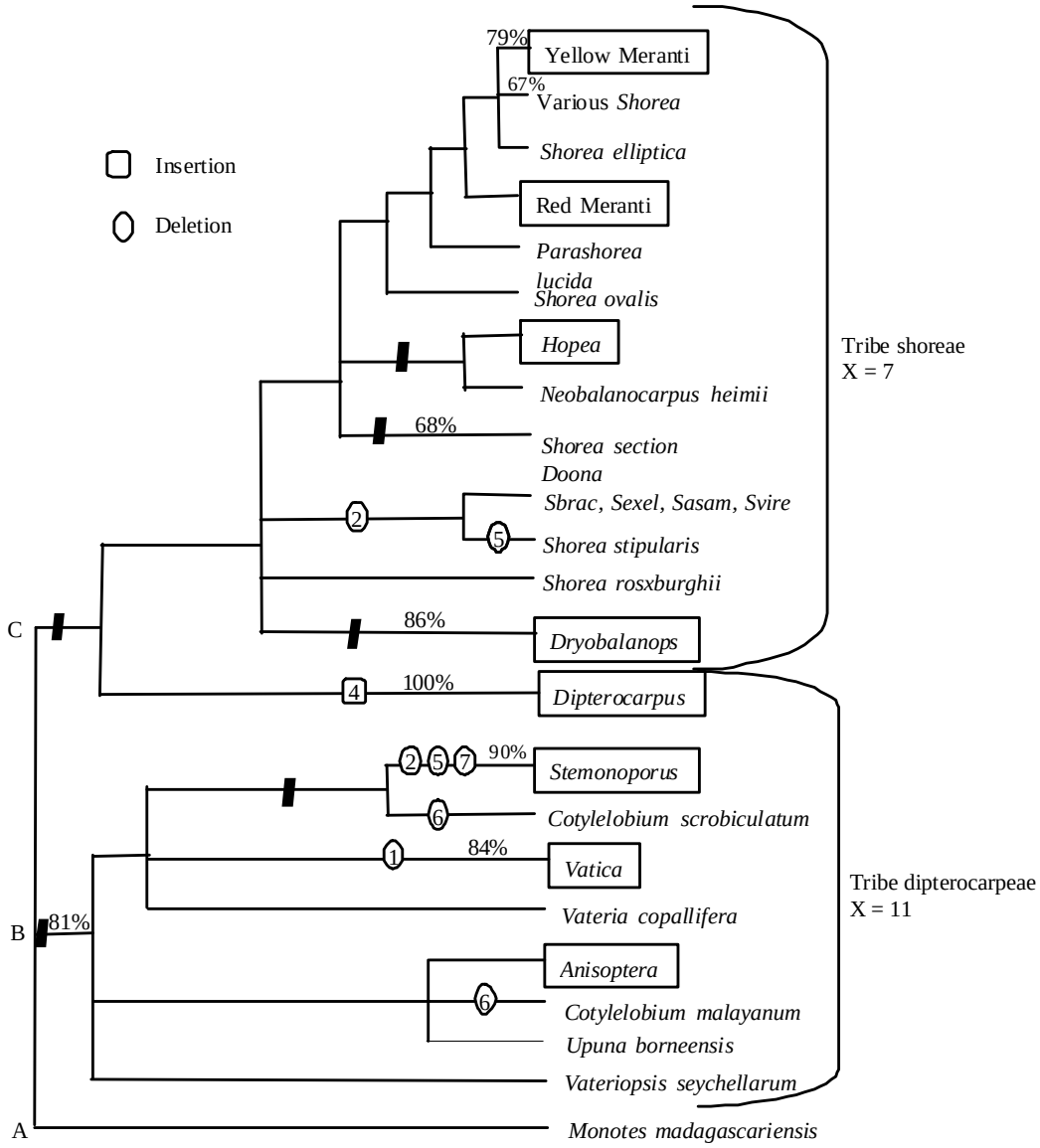

Figure 6. Phylogenetic tree of 110 species of Dipterocarpaceae based on *trn*L intron sequences by including structural mutations. Thick lines are branches appear in strict concensus trees. Taxa in boxes contain all of their species members included in the analysis. Bootstrap supports > 50% are above branches.

89%), while Tribe Shoreae (C) did not received any support from bootstrap (Figure 7). Within tribe Dipterocarpeae, only species of *Stemonoporus* that was weakly supported, other genera/species were not supported. Meanwhile, within Tribe Shoreae only *Shorea* section *Doona* and *Dryobalanops* were weakly supported (61 and 76% respectively).

DISCUSSION

The common practice for phylogenetic reconstruction using molecular evidences is to set foundation of the study on the basis of sequence homology by performing alignment of DNA sequences. Variations within the data set might due to base substitution and/or indel event. The consequence of assigning indels within alignment is length polymorphism (length mutation) within the data set to which secondary structures can be built upon. Secondary structures of *trn*L intron was often built to infer positional homology, for example in Annonaceae (Pirie *et al.* 2007). This was important because inclusion of homoplasious indels into the data set it can be misleading, thus producing incorrect phylogenetic tree. Examination through diagnostic characters (Table 2, Homoplasy Index/HI) revealed that none of the characters within the stem loop structures were homoplasious. Thus these characters were properly suit to be included within a phylogenetic analysis.

On the other hand, the existence of such structures is also useful when such structure is consistently found within certain taxonomic level so that they can be used as molecular marker to detect

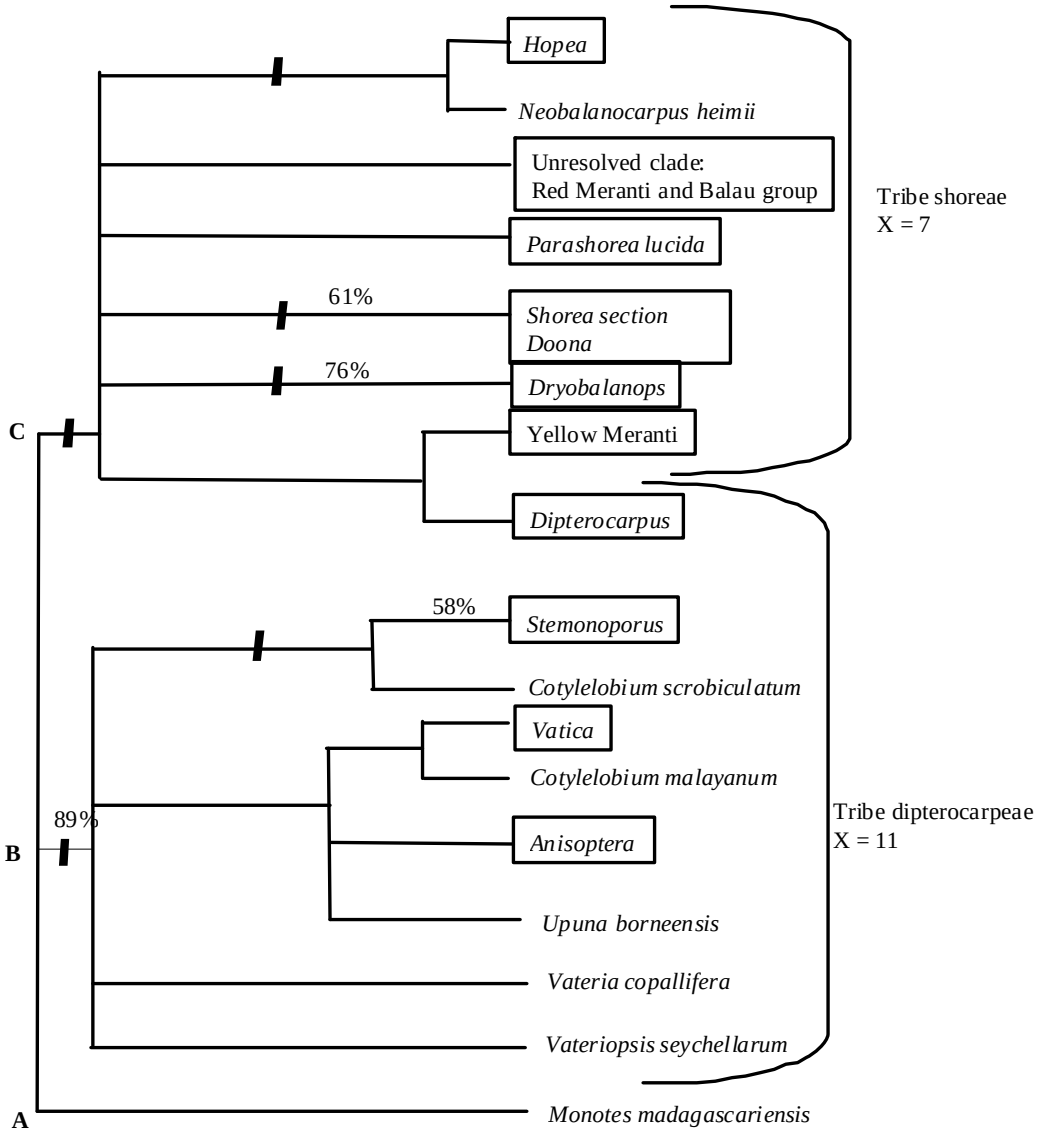

Figure 7. Phylogenetic tree of 110 species of Dipterocarpaceae based on *trn*L intron sequences by excluding structural mutations. Thick lines are branches appear in strict concensus trees. Taxa in boxes contain all of their species members included in the analysis. Bootstrap supports >50% are above branches.

variation at certain taxonomic levels. In this study, inclusion of structural mutations within the data set provided more robust topology for clade C (Figure 6 & 7). The resolved branch includes *Parashorea lucida*, *Shorea* section *Doona*, Red Meranti, Balau, and *Dryobalanops*.

Several classification systems of Dipterocarpaceae were recognized, *i.e.* on the basis of timber grouping (Symington 1943), anatomy (Maury-Lechon & Curtet 1998) and natural group Ashton (1982). The accepted classification system (Ashton 1982) divided this family into 3 sub-families, Dipterocarpoideae (in Asia), Monotoideae (in Africa) and Pakaramoideae (Guayana and Africa). The Asian Dipterocarpoideae contributed the largest number of species within the family. The subfamily Dipterocarpoideae is further divided into two tribes based on the basic chromosome number: 1) tribe Dipterocarpae (x = 11) consisted of genus *Dipterocarpus, Anisoptera, Upuna, Cotylelobium, Vatica, Stemonoporus, Vateria,* and *Vateriopsis*; 2) tribe Shoreae (x = 7) comprises *Dryobalanops, Parashorea, Neobalanocarpus, Shorea,* and *Hopea*. Recent molecular phylogenetic studies of the family using multi cp regions have two different findings in regard to tribal division of subfamily Dipterocarpoideae. The fist was the polyphyly of tribe Dipterocarpaeae and the monophyly of tribe Shoreae (Tsumura *et al.* 1996; Kajita *et al.* 1998; Gamage *et al.* 2006) and the vice versa: tribe Dipterocarpaeae is monophyletic and tribe Shoreae is polyphyletic (Indrioko *et al.* 2006). Indrioko *et al.* 2006 used PCR-RFLP of 17 cp regions, while others employed direct DNA sequencing of some cp genes. These may contributed

Table 2. Character diagnostics for parsimony informative indels. Constant characters are not shown. Location of SL1: 70-105 bp, SL2: 153-171, SL3: 257-328, SL4: 360-386

Char. No.	Tree steps	RI	RC	HI	G-fit
70	1	0/0	0/0	0.000	1.000
79	3	0.000	0.000	0.333	0.750
80	1	0/0	0/0	0.000	1.000
81	1	0/0	0/0	0.000	1.000
82	1	0/0	0/0	0.000	1.000
85	1	0/0	0/0	0.000	1.000
86	2	0/0	0/0	0.000	1.000
87	1	1.000	1.000	0.000	1.000
88	1	0/0	0/0	0.000	1.000
Char. No.	Tree steps	RI	RC	HI	G-fit
89	1	0/0	0/0	0.000	1.000
95	2	0.800	0.400	0.500	0.750
97	1	0/0	0/0	0.000	1.000
99	1	0/0	0/0	0.000	1.000
153	1	1.000	1.000	0.000	1.000
160	3	0.000	0.000	0.333	0.750
162	1	0/0	0/0	0.000	1.000
165	1	0/0	0/0	0.000	1.000
169	1	0/0	0/0	0.000	1.000
Char. No.	Tree steps	RI	RC	HI	G-fit
170	2	0.000	0.000	0.500	0.750
258	1	0/0	0/0	0.000	1.000
261	1	0/0	0/0	0.000	1.000
264	2	0.500	0.250	0.500	0.750
265	1	0/0	0/0	0.000	1.000
269	2	0/0	0/0	0.000	1.000
270	5	0.912	0.365	0.600	0.500
271	2	0.976	0.488	0.500	0.750
277	1	0/0	0/0	0.000	1.000
279	1	0/0	0/0	0.000	1.000
308	1	0/0	0/0	0.000	1.000
309	1	1.000	1.000	0.000	1.000
317	1	0/0	0/0	0.000	1.000
318	1	0/0	0/0	0.000	1.000
320	1	0/0	0/0	0.000	1.000
321	1	0/0	0/0	0.000	1.000
324	1	0/0	0/0	0.000	1.000
Char. No.	Tree steps	RI	RC	HI	G-fit
325	1	0/0	0/0	0.000	1.000
360	1	0/0	0/0	0.000	1.000
363	1	0/0	0/0	0.000	1.000
366	1	0/0	0/0	0.000	1.000
369	1	0/0	0/0	0.000	1.000
372	1	1.000	1.000	0.000	1.000
373	1	0/0	0/0	0.000	1.000
375	1	1.000	1.000	0.000	1.000
380	2	1.000	1.000	0.000	1.000
383	1	1.000	1.000	0.000	1.000
385	2	1.000	1.000	0.000	1.000
525	1	1.000	1.000	0.000	1.000
526	5	0.750	0.150	0.800	0.429
527	1	0/0	0/0	0.000	1.000
528	1	1.000	1.000	0.000	1.000
529	2	0.889	0.444	0.500	0.750
530	2	0.000	0.000	0.500	0.750
531	1	1.000	1.000	0.000	1.000

to the major difference on their results. Second was the inclusion of *Parashorea* within *Shorea* and the monotypic genus *Neobalanopcarpus heimii* within *Hopea*. Not only of these molecular studies (Yulita *et al.* 2005, Indrioko *et al.* 2006; Gamage *et al.* 2006; Tsumura *et al*. 2007) suggested this findings, Symington (1943) has earlier suggested to include *Parashorea* within *Shorea* due to many similarities on morphological traits.

The phylogenetic inference resulting from this study only came from 59 parsimony informative characters but the results of this present study was in accordance to the first finding in that the major groupings tend to follow tribal division to which tribe Dipterocarpae was polyphyletic and tribe Shoreae is monophyletic. The polyphyletic of tribe Dipterocarpeae was caused by the placement of genus *Dipterocarpus* within tribe Shoreae. Examination of SL structures found that there was a large insertion within *Dipterocarpus* located in SL 3. This large insertion is a repeat of 14 nucleotides (GAUUUAUAUUUUUU) exclusively present only in *Dipterocarpus* that may have evolved independently within *Dipterocarpus* (Yulita 2007). Similar findings also suggested by Vijverberg and Bachmann (1999) that structural mutation <1000 bp may have been repeated independent origin of closely related taxa in *Microseris* (Asteraceae). The unresolved polytomy feature in *Dryobalanops* found in previous studies (Dayanandan *et al.* 1999; Yulita *et al.* 2005, Indrioko *et al*. 2006) was well resolved in this study *Dryobalanops* was well supported by 86% BV and 76% BV respectively (Figure 6 & 7). *Dryobalanops* have morphological features (wood anatomy, pollen and floral aestovations) resembled tribe Shoreae and Dipterocarpeae (Maury-Lechon & Curtet 1998). *Dryobalanops* even received 100% support from bootstrap analysis (Gamage *et al.* 2006) when they included more cp genes (*trn*L-F and *mat*K). In addition, the phyletic nature of long debated complex genera, *Shorea* and *Hopea*, was also in accordance to previous studies (Yulita *et al.* 2005; Kamiya *et al.* 2005; Indrioko *et al.* 2006) in which both genera was to form a potential monophyletic group. This could indicated that intron *trn*L consisted of DNA sequences that was evolutionary well preserved. Borsch *et al.* (2003) have demonstrated that the secondary structure of the *trn*L intron is highly conseved in basal Angiospermae, in that only 20% of the 95 posisitions corresponding to proposed stem structures were variable across their study group. Intron *trn*L was suggested to have been present in the cyanobacterial ancestor of the plastid lineages of Rhodophyta, Chlorophyta (Besendahl *et al.* 2000) to

different orders of flowering plants (Bakker *et al.* 2000).

The results from this study was therefore indicated that indels of *trn*L intron in Dipterocarpaceae was of no homoplasious. Similarity of results obtained from this present study to the previous studies that included more cp genes may indicated that DNA sequence of *trn*L intron contained phylogenetic signals that was sufficiently used to reconstruct phylogeny of the subfamily Dipterocarpoideae.

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